Gender differences in antioxidant properties, phenotypic plasticity and freeze tolerance in Seabuckthorn (*Hippophae rhamnoides* L.) along an altitudinal gradient in trans-Himalayan Ladakh, India

SYNOPSIS

Submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy In

Biotechnology

By

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April 2017

Introduction:

Seabuckthorn (*Hippophae rhamnoides* L.) is an ecologically and economically important thorny shrub, which is widely distributed in trans-Himalayan Ladakh region. The species is a dioecious plant pollinated by wind. Once considered a thorny menace, SBT is now being seen as a means for sustainable development of the trans-Himalayan region. The shrub serves as a storehouse for researchers in the field of biotechnology, neutraceutical, pharmaceutical, cosmetic and environmental sciences (Stobdan et al., 2008).

Traditional uses: People living in trans-Himalayan region have developed mastery in judicious use of SBT resources. Every part of the plant is traditionally used for a variety of purposes. Growing of SBT for fencing around agricultural fields is known to improve soil fertility. There are over a hundred popular SBT-based formulations in various pharmacopoeias of *Sowa Rigpa* (Tibetan medicine), which is being practiced in Ladakh. Despite the importance of SBT in the lives of people the age old knowledge of its usage is slowly declining due to opening of the region to the modern world, and availability of alternatives from other parts of the world.

Bioactive content: SBT extracts possess high phenolic content in terms of GAE/100g DW (1666-13769) (Korekar et al., 2011). The mean TPC is highest in seeds (11148) followed by stem bark (10469), leaves (6330) and pulp (3579 mg GAE/100g DW). Further, antioxidant capacity by FRAP assay shows that the stem bark possess maximum antioxidant capacity (16.83) followed by seeds (15.26), leaves (12.73) and pulp (12.61 mM $FeSO_4$). Leaf of SBT also known for antioxidant, immuno- modulatory and anti-cancer activity, anti-inflamatory, hepatoprotective activities, anti-tumor, adaptogenic, hypoxia, cardioprotective, anti atherogenic effects, anti dengue, anti-rabies (Ramachandran et al., 1990;Geetha et al., 2002 , Geetha et al., 2003 , Cheng et al., 2003; Geetha et al., 2005; Ganju et al., 2005; Hibasami et

al., 2005; Geetha et al., 2008; Stobdan et al., 2008; Maheshwari et al., 2011; Saggu and Kumar 2008; Narayanan et al., 2005; Sharma et al., 2014; Jayashankar et al., 2016).

Propagation: There is a growing interest in the vegetative propagation of SBT primarily due to an increasing commercial demand for the plant. Successful plantation of the offspring of elite shrubs will require efficient propagation system in place to mass propagate superior genotypes. Methods have been standardized for propagation of hardwood stem cuttings of the size of a pencil thickness (5-10 mm diameter) from SBT (Dwivedi et al., 2002; Raj et al., 2013). But this method has two major drawbacks for an efficient propagation. First, propagation by a pencil thick hardwood cutting results in fewer cuttings per plant, secondly the rooting success rate is low. Therefore, there is need to developed an improved method for yield of larger number of cuttings with higher success rate. Micropropagation of SBT has been standardized (Dwivedi et al., 2006) but it becomes farmer's unfriendly.

Gender difference: Sex-related differences are still poorly understood, and more physiological studies and diversity of plant species that allow comparative analyses are needed. In some species females are more stress tolerant in than males, and the opposite in others (Juvany et al., 2015). Limited studies have been carried on sex-related differences in antioxidant contents in SBT leaves. Górnaś et al. (2014) studied lipophilic antioxidants in mixed SBT samples of 10 females and 10 males. It was found that lipophilic antioxidant is higher in male as compared to female leaves. In contrast, Šnē et al. (2013) reported higher total phenolics and antioxidant in female SBT leaves. The antioxidant compounds viz. α-tocopherol, β-tocopherol, γ-tocopherol, plastochromanol-8 and β-carotene were observed to be higher in female than in male SBT leaves (Górna et al.,2014).Gender remains in equal ratio in normal environment but are biased to male population in stressful condition (Li et al., 2013).

SBT is a dioecious plant and gender differences in antioxidant properties, phenotypic plasticity and freezing tolerance may differ between male and female individuals due to greater reproductive effort in females. The cost of reproduction involves prioritization of resources in fruit development rather than in vegetative growth or protection in females. We expected to find gender differences and strong altitudinal variation in antioxidant properties, phenotypic plasticity and freeze tolerance. In view of the dioecious nature of SBT, the present study has been conducted with the following objectives:

1. Development of an improved propagation method of Seabuckthorn (*Hippophae rhamnoides* L.) by stem cutting
2. Sexual differences and seasonal variation in total phenolics and antioxidant capacities in *Hippophae rhamnoides* leaves.
3. Gender differences in phenotypic plasticity and adaptive response of Seabuckthorn (*Hippophae rhamnoides* L.) along an altitudinal gradient in trans-Himalaya.
4. Gender-specific seasonal pattern and altitudinal variation in freeze tolerance responses in *Hippophae rhamnoides*.
5. SSR markers based genetic diversity characterization and marker-trait correlation for antioxidant properties in male and female populations of *Hippophae rhamnoides.*

Objective I

Development of an improved method for propagation of Seabuckthorn (*Hippophae rhamnoides* L.) by cuttings

Hardwood cuttings are more often used in propagation of deciduous woody plants as one of the cheapest and easiest methods of vegetative propagation. Typically, they require less or no special equipment during rooting, which can be readily performed in nurseries (Exadaktylou et al., 2009). Recent interest in the vegetative propagation of SBT arises primarily because there is increasing demand for SBT. Currently, there is a body of information concerning the propagation of pencil thickness (5-10 mm diameter) hardwood stem cuttings from SBT (Singh et al., 1995; Dwivedi et al., 2002; Rao et al., 2010 and Raj et al., 2013). However, propagation by pencil thickness hardwood cutting results in fewer cuttings per plant. Besides, rooting success rate through pencil thickness cutting is low. Micropropagation of SBT is possible but it requires high-tech facilities and well-trained personnel, which is lacking in most places where SBT can be grown.

Till date, there is much information concerning the propagation of pencil thickness (5-10 mm diameter) hardwood stem cuttings from SBT. However, the influence of mulching, shading, spacing and cutting thickness on rooting success and growth has received almost no attention. Therefore, the objective of the present study was to develop an improved propagation method and to study the effect of plastic mulching, coloured shade net, spacing and cutting thickness on rooting success and growth of SBT in nurseries.

Result

One year old growth stem cutting (2.9±0.8 mm thickness), which is less than half the thickness of conventional pencil thickness (7-8 mm) hardwood cutting, was studied for vegetative propagation of Seabuckthorn (SBT). Rooting trial tested the effects of 14 media

and six concentration of indole-3-butyric acid treatment. The experiment was designed as a completely randomized block with 14 rooting media × six IBA levels × three replicates of 15 cuttings. One-way ANOVA was performed with the help of 2-sided Tukey's HSD at $P \leq 0.05$ (Table 1.1). All statistical analysis was performed using SPSS for Windows 17.0 version. Over 94% rooting was observed in all the media tested. The mean rooting percentage was 97.6±2.2. Exogenous auxin treatments lack any benefit and rooting was over 97% without hormonal treatment. Seven-fold more number of cuttings was obtained per plant as compared to pencil thickness hardwood cuttings, which is the conventional method of SBT propagation. From a mature shrub, the mean number of one year old growth cuttings (2.9±0.8 mm diameter) that can be used for vegetative propagation, as discussed in the present study, was 151.8±106.6. In comparison, 21.3±8.3 number of pencil thickness (7.0±0.3 mm diameter) cuttings was obtained per shrub. Therefore, 7-fold more number of cuttings per plant as compared to pencil thickness hardwood cuttings demonstrated the advantage of using one year old growth cutting for vegetative propagation of SBT.

Use of silver black plastic mulching film resulted in 10% higher rooting success and significant plant growth in pencil thickness (5-10 mm diameter) cuttings. Suppression of weed emergence by the plastic mulch resulted in 75.8% time saving in manual weeding by farm workers. Reduction in light intensity by 66% using green shade net resulted in significant reduction in rooting and growth of nursery plants, which agrees with previous results in shade-intolerant pioneer *Pinus halepensis* where shading treatment produced low quality seedlings with poor root development (Puértolas et al., 2009). Three different spacing between cuttings did not show significant difference in rooting and growth related parameters suggesting that cuttings can be planted denser (3"×3") under mulching to get higher number of nursery stock per unit area. Cutting thickness showed significant effect on rooting success. Highest rooting percentage was observed in pencil thickness cuttings

(7.5±1.6 mm diameter) followed by cuttings with 2.9±0.8 mm and 11.3±1.7 mm basal diameter. No significant gender difference in rooting success was observed in mulched condition (Table 1.2). However, significantly higher shoot length, number of primary root and basal diameter of secondary root was observed in male as compared to female plants. In contrast, (Dhyani et al., 2012) reported higher rooting success and number of roots per cuttings in female cuttings than in male donor plants in *H. salicifolia*. The result of the present study could facilitate establishment of a vegetative propagation method wherein faster growth and larger number of cuttings can be propagated with higher rooting success rate.

The result of the present study could facilitate establishment of a vegetative propagation method wherein larger number of cuttings per plant can be propagated with higher rooting success rate without the need for exogenous application of rooting hormone.

Table 1.1: Combined effect of different rooting media and rooting hormone (IBA) concentration on rooting percentage and growth parameters after 13 weeks

Treatment		Rooting %	Shoot length per cutting (mm)	Number of shoot per cutting	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of the longest primary root (mm)	Length of the longest secondary root (mm)	Diameter of basal end of primary root (mm)
Rooting Media	M1	93.9±1.5^{a}	58.4±17.0^{bc}	7.6±3.0^{a}	22.7±6.1^{ab}	7.1±3.5^{ab}	69.1±27.7^{abc}	148.1±56.2^{ab}	48.2±16.1^{def}	1.2±0.2^{ab}
	M2	98.7±2.3^{b}	60.3±23.9^{bc}	10.4±2.5^{a}	21.6±7.9^{ab}	7.0±2.5^{ab}	85.5±39.5^{bc}	**205.8±58.7^{b}**	**64.0±33.8^{f}**	1.1±0.2^{ab}
	M3	97.8±3.2^{ab}	52.2±17.6^{abc}	7.8±3.0^{a}	21.4±7.9^{ab}	6.7±3.1^{ab}	65.9±26.4^{abc}	144.5±69.4^{ab}	47.4±15.9^{cdef}	1.4±1.1^{ab}
	M4	97.9±2.0^{ab}	45.3±16.3^{ab}	8.9±2.2^{a}	20.1±3.9^{ab}	5.9±2.5^{a}	71.8±28.8^{abc}	141.0±65.7^{ab}	35.7±16.9^{abcde}	1.0±0.2^{ab}
	M5	95.3±4.5^{ab}	49.0±19.2^{abc}	7.9±4.0^{a}	18.2±5.3^{ab}	5.1±2.5^{a}	58.2±23.9^{ab}	142.0±70.5^{ab}	22.9±12.0^{a}	1.0±0.3^{a}
	M6	96.1±3.5^{ab}	48.9±16.1^{abc}	7.9±2.8^{a}	18.6±5.3^{ab}	5.1±2.3^{a}	51.3±22.8^{a}	105.7±50.2^{a}	25.7±17.7^{ab}	1.1±0.3^{ab}
	M7	97.4±2.1^{ab}	50.3±13.8^{abc}	9.6±3.5^{a}	19.5±4.0^{ab}	5.3±2.1^{a}	42.8±17.0^{a}	116.5±46.6^{a}	30.7±12.1^{abcd}	1.3±0.4^{ab}
	M8	97.2±2.2^{ab}	58.1±15.8^{bc}	8.4±3.0^{a}	21.1±3.7^{ab}	6.2±2.2^{ab}	68.0±25.7^{abc}	127.6±64.4^{ab}	26.4±10.0^{ab}	1.2±0.2^{ab}
	M9	97.4±2.1^{ab}	**67.9±21.4^{c}**	9.4±2.2^{a}	23.1±7.8^{b}	6.8±2.4^{ab}	65.9±23.3^{abc}	176.8±73.5^{ab}	46.4±25.6^{bcdef}	1.0±0.2^{a}
	M10	97.8±2.0^{ab}	65.3±24.2^{bc}	8.6±2.6^{a}	21.3±7.7^{ab}	7.5±3.^{ab}	69.3±31.6^{abc}	187.3±111.4^{ab}	54.9±21.7^{ef}	1.2±0.3^{ab}
	M11	98.9±2.1^{b}	37.1±16.4^{a}	8.9±2.8^{a}	17.3±4.6^{ab}	5.4±2.7^{a}	45.1±22.6^{a}	183.4±163.1^{ab}	27.3±12.6^{abc}	1.4±0.4^{ab}
	M12	98.9±1.1^{b}	59.4±15.8^{bc}	9.8±2.3^{a}	**23.3±4.6^{b}**	**9.5±4.6^{b}**	74.2±24.2^{abc}	173.2±71.4^{ab}	37.1±13.3^{abcde}	**1.5±0.3^{b}**
	M13	**99.8±0.2^{b}**	33.2±7.8^{a}	**10.6±3.3^{a}**	16.5±3.1^{a}	6.1±2.4^{ab}	49.9±15.3^{a}	151.6±45.9^{ab}	30.8±15.7^{abcd}	1.4±0.3^{ab}
	M14	99.1±1.0^{b}	57.8±19.5^{bc}	10.4±2.3^{a}	21.2±3.1^{ab}	7.7±4.1^{ab}	**97.4±53.5^{c}**	124.3±68.0^{ab}	35.5±20.4^{abcde}	1.4±0.4^{ab}
	Mean	97.6±2.2	53.1±19.9	9.0±3.0	20.4±5.9	6.5±3.1	64.8±32.1	152.0±81.4	38.1±21.5	1.2±0.4
IBA (ppm)	0	97.4±2.9^{xy}	48.7±20.2^{x}	9.2±3.2^{xy}	19.7±7.0^{x}	5.9±2.6^{x}	59.9±29.9^{x}	158.1±112.6^{x}	38.9±27.6^{x}	1.1±0.4^{x}
	200	96.9±2.5^{xy}	**54.8±18.1^{x}**	8.9±3.5^{xy}	20.8±4.9^{x}	6.4±3.1^{x}	62.1±28.5^{x}	143.1±55.6^{x}	35.9±18.8^{x}	1.2±0.3^{x}
	500	95.5±4.4^{x}	54.5±20.8^{a}	9.5±2.5^{xy}	20.6±6.6^{x}	6.5±3.2^{x}	67.5±31.2^{x}	**161.0±80.5^{x}**	40.1±22.6^{x}	1.2±0.3^{x}
	1000	**98.8±0.9^{y}**	54.4±20.1^{x}	8.0±3.0^{x}	**21.4±5.9^{x}**	6.4±3.8^{x}	66.6±40.5^{x}	160.2±72.6^{x}	**42.4±23.8^{x}**	1.3±0.3^{x}
	2000	98.7±1.1^{y}	53.9±18.2^{x}	**10.0±2.8^{y}**	21.0±5.0^{x}	6.7±2.4^{x}	66.0±26.1^{x}	159.9±87.0^{x}	36.2±16.5^{x}	1.2±0.3^{x}
	5000	98.3±1.4^{y}	52.3±22.5^{x}	8.5±2.4^{xy}	19.0±5.4^{x}	**7.3±3.5^{x}**	**69.9±35.1^{x}**	129.6±67.5^{x}	35.0±18.2^{x}	**1.4±0.8^{x}**
	Mean	97.6±2.2	53.1±20.0	9.0±3.0	20.4±5.9	6.5±3.1	65.3±32.1	152.0±81.4	38.1±21.5	1.2±0.4

M1: peat moss : perlite (2:1), **M2:** peat moss : perlite (1:2), **M3:** peat moss : vermiculite (1:1), **M4:** peat moss : manure (1:1), **M5:** peat moss : soil (2:1), **M6:** soil : manure (2:1), **M7:** sand : manure (2:1), **M8:** sand : soil (2:1), **M9:** vermiculite, **M10:** peat moss, **M11:** soil, **M12:** sand, **M13:** manure, and **M14:** perlite.

Values represented as mean±SD; for each column, different lowercase letters within each treatment indicate significantly different at $P \leq 0.05$, as measured by 2-sided Tukey's HSD

Table 1.2: Effect of mulching, shading, gender of donor plant, spacing and cutting thickness on rooting percentage and growth of Seabuckthorn cuttings in nursery

Treatment	Rooting %	Number of shoot per cutting	Average shoot length of cutting (cm)	Number of leaf per shoot	Number of primary root per cutting	Number of secondary root per cutting	Length of longest primary root (mm)	Length of longest secondary root (mm)	Diameter of basal end of primary root (mm)	Diameter of basal end of secondary root (mm)
A. Mulching, shading and gender of donor plant										
Mulch (female)	84.0±5.3^{b}	7.2±2.0^{a}	20.3±3.8^{bc}	40.1±7.3^{c}	8.3±3.3^{ab}	60.8±12.2^{a}	63.3±27.4^{b}	25.8±3.4^{b}	3.5±0.6^{a}	1.6±0.5^{ab}
Non-mulch	75.7±4.1^{ab}	5.7±0.6^{a}	13.5±1.7^{ab}	28.0±6.1^{ab}	6.0±1.7^{ab}	38.3±11.6^{abc}	34.8±3.6^{ab}	14.1±6.6^{ab}	2.2±0.3^{a}	0.8±0.1^{a}
Mulch + shade	78.3±0.6^{ab}	5.8±1.2^{a}	15.6±2.1^{ab}	34.8±6.1^{bc}	7.5±2.1^{ab}	35.0±9.8^{ab}	32.0±18.2^{ab}	10.8±5.4^{a}	2.1±0.6^{a}	0.7±0.3^{a}
Non-mulch + shade	72.7±1.1^{a}	4.0±1.8^{a}	9.3±6.5^{a}	17.7±6.6^{a}	4.0±1.8^{a}	23.5±14.9^{a}	16.7±10.2^{a}	6.4±2.4^{a}	2.2±1.4^{a}	0.8±0.4^{a}
Mulch (male)	84.7±2.5^{b}	5.3±2.2^{a}	28.1±7.5^{c}	42.9±2.4^{c}	9.0±1.3^{b}	55.7±16.3^{bc}	63.1±16.2^{b}	25.2±11.6^{b}	3.5±0.4^{a}	2.1±0.6^{b}
Mean	79.9±3.5	5.9±1.7	17.9±4.7	34.0±6.6	7.2±2.3	45.8±13.7	45.7±18.5	18.0±6.1	2.8±0.7	1.3±0.4
B. Spacing between plant to plant and row to row (mulched)										
6"×6"	83.5±1.1^{a}	6.7±1.0^{a}	23.9±4.9^{a}	43.0±12.1^{a}	8.2±3.8^{a}	62.5±8.4^{a}	55.6±4.1^{a}	22.7±2.8^{ab}	4.1±0.6^{a}	1.4±0.3^{a}
3"×3"	84.0±5.3^{a}	7.2±2.0^{a}	20.3±3.8^{a}	40.1±7.3^{a}	8.3±3.3^{a}	60.8±12.2^{a}	63.3±27.4^{a}	25.8±3.4^{ab}	3.5±0.6^{a}	1.6±0.5^{a}
*Triangular	84.0±1.2^{a}	5.8±0.8^{a}	19.0±3.1^{a}	40.5±4.8^{a}	7.5±1.8^{a}	46.2±13.2^{a}	66.1±20.2^{a}	12.9±5.4^{a}	3.7±0.4^{a}	1.3±0.5^{a}
Mean	83.8±2.8	6.6±1.4	21.1±4.3	41.2±8.2	8.0±2.9	56.5±13.2^{a}	61.6±19.1	20.4±6.8	3.8±0.6	1.4±0.4
C. Cutting thickness: > Pencil thickness (11.32±1.7 mm); Pencil thickness (7.45±1.6 mm); <Pencil thickness (2.9±0.8 mm) (non-mulched)										
> Pencil thickness	50.4±6.6^{a}	6.7±2.1^{a}	13.2±6.8^{a}	27.5±9.5^{a}	7.3±3.5^{a}	36.3±2.1^{a}	50.6±21.3^{a}	10.7±3.0^{a}	2.2±0.3^{a}	0.8±0.2^{a}
Pencil thickness	75.7±4.1^{ab}	5.7±0.6^{a}	13.5±1.6^{a}	28.0±6.1^{a}	6.0±1.7^{a}	38.3±11.6^{a}	34.8±3.6^{a}	14.1±6.6^{a}	2.2±0.3^{a}	0.8±0.1^{a}
<Pencil thickness	65.0±1.7^{ab}	5.3±0.6^{a}	12.4±0.5^{a}	25.5±2.1^{a}	6.3±0.6^{a}	33.7±2.1^{a}	37.3±1.2^{a}	19.8±3.4^{a}	2.4±0.4^{a}	0.8±0.2^{a}
Mean	63.7±5.7	5.9±1.3	13.0±3.6	27.0±5.9	6.6±2.7	36.1±6.3	40.9±13.1	14.9±5.6	2.3±0.3	0.8±0.2

Values represented as mean±SD of 30 cuttings; for each column, different lowercase letters within each treatment (A, B and C) indicate significantly different at P≤0.05, as measured by 2-sided Tukey's HSD; *Triangular: additional one cutting in the centre of 6"×6".

Objective II

Sexual differences and seasonal variations in total phenolics and antioxidant properties in *Hippophae rhamnoides* leaves

Although the nutritional and medicinal properties of SBT berries are usually the focus of attention, SBT leaf has been receiving much attention in recent years for its medicinal and therapeutic applications. Many of these activities are attributed to high antioxidant capacity including the phenolics. Dried SBT leaves are used for tea and processed for nutraceuticals products. Antioxidant properties of SBT leaves have been extensively studied (Arora et al., 2012; Maheshwari et al., 2011; Guan et al., 2005 and Korekar et al., 2011). However, SBT is a dioecious plant and hence sexual differences in presence of health promoting compounds in its vegetative parts is expected due to greater reproductive effort in females. Limited studies have been carried on sex-related differences in antioxidant activity and phenolic content in SBT leaves. Górnaś et al (2014) studied lipophilic antioxidants in mixed SBT samples of 10 females and 10 males. It was found that lipophilic antioxidant is higher in male as compared to female leaves. In contrast, Šnē et al. (2013) reported higher total phenolics and antioxidant in female SBT leaves.

Result

Leaves of six natural populations (Table 2.1) of SBT, which comprised 200 plants (100 males, 100 females) from the trans-Himalaya were harvested, and their methanolic and acetone extracts were studied for TPC and TAC (Table 2.2). Males exhibit significantly higher TPC (100.8±23.9 mg GAE/g DW) than females (95.0±23.8 mg GAE/g DW). Similarly, ferric reducing activity was significantly higher in males (6.5±1.1 Fe^{2+} mmol/g DW) than females (6.1±1.2 Fe^{2+} mmol/g DW) (Table 2.2). Significant increase in TPC was observed in male leaves from July to October followed by a significant decrease in

November (Fig 2.1). However, a trend of increase in TPC up to August and a steady decline thereafter was observed in leaves of female SBT. Similarly a trend of an increasing TAC was observed in both the sexes but female leaves were observed to be on an increasing TAC from July to October. Male SBT leaves exhibit higher TPC and TAC than females; October is the best time for harvesting SBT leaves and; SBT leaves contain significantly higher hydrophilic than lipophilic phenolics and antioxidants.

Table 2.1: Location of the populations studied

Sampling Locations	Population ID	Latitude (N)	Longitude (E)	Altitude (m) ASL	Sample size (numbers)	
					Male	Female
Spituk	SPT	34º07′7″	77º30′4″	3203±5.6	20	20
Chuchot	CHU	34º05′4″	77º35′9″	3239±5.0	17	17
Shey	SHY	34º04′1″	77º37′7″	3260±4.6	17	17
Phyang	PHY	34º11′5″	77º30′1″	3636±49.6	16	16
Horzey	HOR	34º12′1″	77º35′3″	3812±24.8	15	15
Sakti	SKT	34º02′1″	77º48′6″	3885±37.3	15	15

Table 2.2: Sexual difference in total phenolic content (mg GAE/g DW) of *H. rhamnoides* (100 males, 100 females) leaves

Population ID	Male leaves					Female leaves				
	Hydrophilic	Lipophilic	Combined[1]			Hydrophilic	Lipophilic	Combined[1]		
	Mean±SD			Min	Max	Mean±SD			Min	Max
SPT	101.60±20.47^{b}	2.27±0.26^{bc}	103.86±20.58^{b}	61.53	142.45	107.76±19.95^{c}	2.27±0.40^{bc}	110.03±20.05^{c}	76.72	165.83
CHU	123.88±21.83^{c***}	2.19±0.22^{ab}	126.07±21.84^{c***}	93.03	173.06	91.67±25.43^{b}	2.95±0.40^{e***}	94.62±25.34^{b}	51.02	152.44
SHY	86.87±18.28^{a***}	2.48±0.40^{c}	89.66±18.90^{a***}	47.19	131.67	74.98±10.95^{a}	2.53±0.49^{d}	77.51±11.15^{a}	51.99	97.03
PHY	93.43±15.18^{ab}	2.03±0.28^{a}	95.46±15.37^{ab}	63.69	124.28	109.59±16.45^{c***}	2.11±0.30^{ab}	111.70±16.70^{c***}	81.86	144.57
HOR	84.71±12.82^{a}	2.49±0.39^{c}	87.21±12.88^{a}	67.04	120.58	94.36±13.65^{b***}	2.38±0.35^{cd}	96.89±14.10^{b***}	73.41	125.42
SKT	99.91±29.63^{b***}	3.12±0.66^{d***}	103.02±29.70^{b***}	58.07	170.36	76.76±26.49^{a}	2.01±0.38^{a}	78.77±26.76^{a}	29.88	132.01
Average	98.24±23.82**	2.41±0.60	100.83±23.92**	47.19	173.06	92.64±23.66	2.37±0.49	95.02±23.82	29.88	165.83

Population ID	Male leaves				Female leaves			
	[1]FRAP ($FeSO_4.7H_2O$ mmol/g DW)			[2]IC_{50} (mg/ml)	[1]FRAP ($FeSO_4.7H_2O$ mmol/g DW)			[2]IC_{50} (mg/ml)
	Hydrophilic	Lipophilic	[3]Combined	[4]Combined	Hydrophilic	Lipophilic	[3]Combined	[4]Combined
SPT	7.07±1.17^{c}	0.13±0.01^{a}	7.21±1.18^{c}	0.34±0.10^{a}	7.21±1.04^{c}	0.14±0.02^{ab}	7.35±1.05^{c}	0.32±0.18^{a}
CHU	7.01±0.61^{c***}	0.14±0.02^{ab}	7.15±0.60^{c***}	0.33±0.03^{a***}	6.05±1.20^{b}	0.18±0.03^{d***}	6.23±1.21^{b}	0.37±0.03^{a}
SHY	5.75±0.71^{ab}	0.15±0.02^{bc}	5.90±0.73^{b}	0.40±0.27^{ab}	5.63±0.62^{ab}	0.17±0.02^{c***}	5.80±0.62^{b}	0.42±0.29^{a}
PHY	6.86±0.63^{c***}	0.16±0.02^{c**}	7.02±0.64^{c***}	0.38±0.05^{ab}	5.54±0.70^{ab}	0.15±0.02^{b}	5.69±0.72^{ab}	0.37±0.03^{a}
HOR	6.09±0.91^{b}	0.15±0.02^{c***}	6.24±0.91^{b}	0.46±0.17^{bc}	6.08±0.82^{b}	0.13±0.02^{a}	6.21±0.83^{b}	0.44±0.13^{a}
SKT	5.28±1.26^{a}	0.15±0.03^{c***}	5.08±1.80^{a}	0.54±0.20^{c}	5.07±1.32^{a}	0.13±0.02^{a}	5.19±1.34^{a}	0.65±0.35^{b}
Average	6.37±1.14***	0.15±0.2	6.51±1.14***	0.41±0.18	5.96±1.19	0.15±0.03	6.11±1.20	0.43±0.23

Sexual difference in total antioxidant capacity of *H. rhamnoides* (100 males, 100 females) leaves

Values represented as mean ± SD

For each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by 2-sided Tukey's HSD between populations

[1]FRAP: Ferric reducing antioxidant potential

[2]IC_{50}: Inhibitory concentration, the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration

[3]Combined: Values of hydrophilic and lipophilic extract combined mathematically

[4]Combined: Values of combined hydrophilic and lipophilic extract measured

**value significantly higher than that of opposite sex at $p \leq 0.01$, *** value significantly higher than that of opposite sex at $p \leq 0.001$

Table 2.3: Seasonal variation in total phenolic content and total antioxidant capacity of *H. rhamnoides* (5 males, 5 females) leaves

Month	Male			Female		
	[1]TPC	[2]FRAP	[3]IC_{50}	[1]TPC	[2]FRAP	[3]IC_{50}
July	66.75±7.15^{a}	4.12±0.20^{a***}	0.52±0.20^{c}	59.97±16.63^{abc}	3.39±0.50^{a}	0.69±0.57^{a}
August	81.59±10.71^{bc**}	5.09±0.49^{b***}	0.26±0.09^{b***}	69.57±13.04^{c}	3.78±0.37^{ab}	0.52±0.16^{a}
September	87.38±6.13^{cd***}	5.62±0.51^{bc***}	0.19±0.08^{ab***}	63.45±8.02^{bc}	4.27±0.32^{c}	0.73±0.16^{a}
October	93.25±7.14^{d***}	6.74±0.57^{d***}	0.12±0.04^{a***}	55.02±8.04^{ab}	4.98±0.33^{d}	0.85±0.63^{a}
November	73.90±10.97^{ab***}	5.68±0.85^{c***}	0.31±0.18^{b***}	51.47±6.60^{a}	4.09±0.47^{bc}	0.94±0.43^{a}
Average	80.57±12.68***	5.45±1.02***	0.28±0.19***	59.89±12.56	4.10±0.66	0.75±0.45

Values represented as mean ± SD

For each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by 2-sided Tukey's HSD between months

[1]TPC: Total phenolic content (mg GAE/g DW)

[2]FRAP: Ferric reducing antioxidant potential ($FeSO_4.7H_2O$ mmol/g DW)

[3]IC_{50}: Inhibitory concentration, the amount of sample extracted into 1 ml solution necessary to decrease by 50% the initial DPPH concentration

**value significantly higher than that of opposite sex at $p \leq 0.01$; *** value significantly higher than that of opposite sex at $p \leq 0.001$

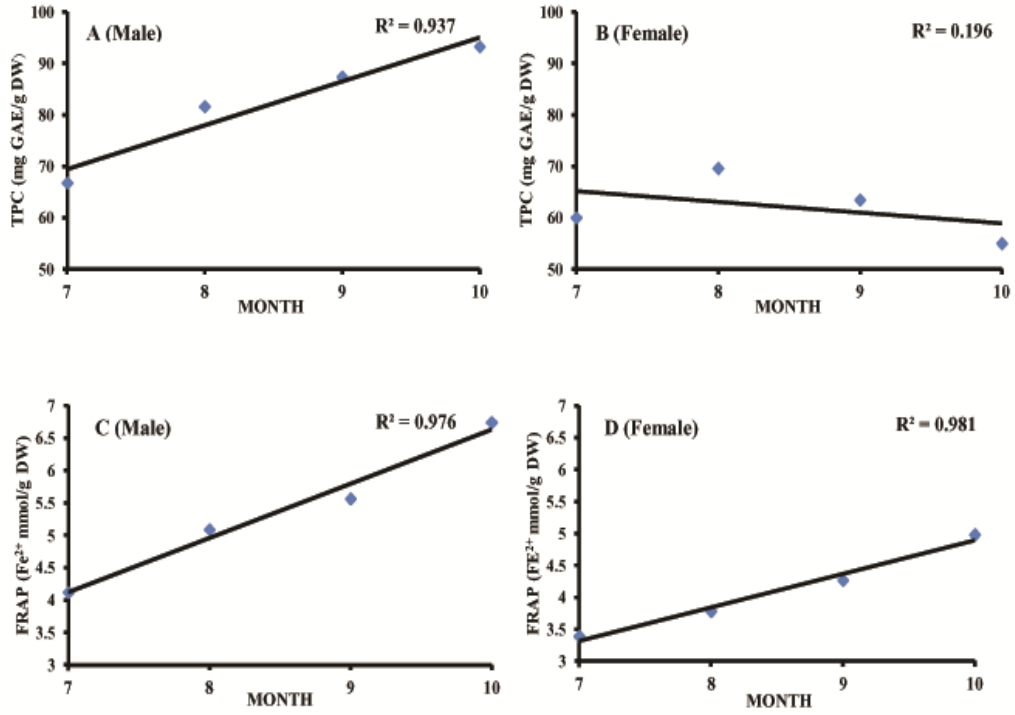

Fig. 2.1. Relation between total phenolic content (A-B) and antioxidant capacity (C-D) in male and female Seabuckthorn leaves with harvest season(July-October).

Objective III:

Gender differences in phenotypic plasticity and adaptive response of *Hippophae rhamnoides* along an altitudinal gradient in trans-Himalaya

Climate change is altering the availability of resources and the conditions that are crucial to plant performance (Nicotra et al., 2010). Over the last few decades, attention is being increasingly focused on evolutionary responses to rapid climate change (Bresson et al., 2011). Plant species can adjust to changing climate through environmentally induced shift in phenotype (phenotypic plasticity), adapt through natural selection (genetic response) or migrate to follow conditions to which they are adapted; these options are not mutually exclusive (Nicotra et al., 2010). However, studies of climate change-induced evolution under simulated and natural climatic conditions have rarely integrated plastic and genetic evolutionary responses (Reusch and Wood, 2007). In dioecious plants, morphological adjustment to climate change may differ between male and female individuals due to greater reproductive effort in females. The cost of reproduction involves prioritization of resources in fruit development rather than in vegetative growth or protection in females. Not accounting for sexual variation could lead to incorrect assessment of a species response to climate change (Jones et al., 1999). However, this aspect has not been studied in detail, particularly in the fragile trans-Himalayan region. Altitudinal gradients in stressful mountain ecosystem provide an ideal experimental opportunity for studying the functional traits of plants in response to abrupt climate change. In mountainous regions, sharp changes in abiotic factors occur over short distances, leading to major changes in the selection pressures acting on plant life history traits (Bresson et al., 2011).

Result

Gender differences in phenotypic variation along altitudinal gradient

Both altitude and gender had a significant impact on leaf morphology of field-grown *H. rhamnoides* (Table 3.2). Leaves become smaller in length and area, but became thicker with decreasing SLA with increasing altitude in both the gender. Effect of altitudinal gradient on leaf thickness was more evident in male. The linear regression showed almost linear (R^2 = 0.82) relation between leaf thickness with altitude in male, but there was no linear relationship in female (R^2 = 0.02) (Table 3.3). Petiole length in males increased significantly with increasing altitude (R^2 = 0.44), but similar pattern was not observed in females (R^2 = 0.04). No significant relationship with altitude was observed for leaf width in both the gender. The chlorophyll contents varied between 'high' and 'low' altitudes, with leaves from higher altitude (3500-4200 m) having more than those from lower altitude (2800-3300 m) in both the gender.

Within each altitude, there was a significant influence of gender on all morphological characters measured. Leaf length, width, thickness, area and chlorophyll contents were higher in males than in females (Table 3.2). The effect of altitude and gender on leaf morphology was supported by results of two-way ANOVA (Table 3.4). Predominant effects of altitude on leaf length (F_1 = 3.8, $p \leq 0.05$), thickness (F_1 = 3.8, $p \leq 0.05$), petiole length (F_1 = 3.3, $p \leq 0.05$), chlorophyll contents (F_1 = 3.0, $p \leq 0.05$), and SLA (F_1 = 12.0, $p \leq 0.001$) was observed. Similarly, significant effect of gender was observed on leaf width (F_1 = 7.0, $p \leq 0.05$), thickness (F_1 = 4.4, $p \leq 0.05$), area (F_1 = 7.5, $p \leq 0.05$) and SLA (F_1 = 7.3, $p \leq 0.01$). Males showed more variability than females in all the leaf morphological traits except for leaf thickness (Table 3.5).

Genetic differentiation in the common-garden experiment

Altitude of plant origin did not have significant impact on leaf morphology of garden-grown *H. rhamnoides* (Table 3.2). No increasing or decreasing trend was observed in leaf length, width, thickness and petiole length with increasing altitude of origin in both the gender. However, a relationship between leaf chlorophyll contents and altitude was observed (R^2 = 0.37) in males, with lower chlorophyll contents in plants from higher altitude origin. Similar relationship was observed between altitude and SLA of garden-grown male plants (R^2 = 0.26), with decreasing SLA with increasing altitude of origin (Table 3.3). However, within each altitude of origin, there was a significant influence of gender on all morphological characters measured. Leaf length, petiole length, leaf area and chlorophyll contents were higher in males than in females (Table 3.2). SLA was significantly higher in females of all altitude of origin. However, two-way ANOVA results did not support significant effect of gender on leaf morphology in garden-grown plants (Table 3.4). A reduced variability in leaf morphology was observed in garden-grown plant as compared to field-grown plant in both the gender (Table 3.5). However, the exceptions were chlorophyll contents and SLA which remained unchanged in one of the gender.

The leaves of the low altitude origin (2800-3300 m) garden-grown plants were smaller in length than those collected from the field in both the gender. However, the opposite trend was observed in plants of higher altitude origin (3500-4200 m). Leaf area of garden-grown male plants was significantly smaller than field-grown plants irrespective of their altitude of origin. However, the trend was not observed in female leaves. Chlorophyll contents of garden-grown leaves remained higher in males of low altitude origin (2800-3300 m) than field-grown plant. However, a significant lowering trend was observed in leaves of garden-grown plants of higher altitude origin (3500-4200 m). In contrast, no such increasing or decreasing trend was observed in females. The SLA of garden-grown male leaves was

lower than those collected from the field. However, in females the trend was observed only in plants of low altitude origin (2800-3300 m).

Table 3.1 Geographical location and sampling sites of *Hippophae rhamnoides* in trans-Himalaya India

Valley/Populations	Sub Populations	Population ID	No. of Samples	Altitude (m asl)	Latitude	Longitude
Indus	Achinathang	ACH	2	3566	34º 50.090"N	76º 63.596"E
	Nurla	NUR	2	3018	34º 17.946"N	76º 59.107"E
	Phey	PHY	2	3179	34º 14.038"N	77º 48.083"E
	Shey	SHY I	4	3232	34º 06.759"N	77º 63.324"E
	Horzey	HOR I	4	3885	34º 20.722"N	77º 60.878"E
	Sakti I	SAK I	2	3927	34º 01.362"N	77º 81.643"E
	Sakti II	SAK II	2	3680	33º 97.161"N	77º 80.290"E
Nubra	Hundar	HUN	2	3077	34º 57.362"N	77º 49.348"E
	Diskit	DIS	2	3119	34º 33.056"N	77º 33.201"E
	Turtuk	TUR I	4	2797	34º 84.804"N	76º 49.712"E
	Panamik	PAN	2	3180	34º 46.739"N	77º 32.449"E
	Khardong	KDG	2	4117	34º 58.164"N	77º 66.294'E
Changthang	Durbuk	DUR	2	3850	34º 09.884"N	78º 12.416"E
	Shayok I	SHK I	2	3740	34º 18.074"N	78º 13.793"E
	Shayok II	SHK II	2	3576	34º 17.232"N	78º 14.603"E

Table 3.2 Interpopulation variability (coefficient of variation) of leaf morphological traits in the field grown and glass house grown *Hippophae rhamnoides*

Variable	Field grown	Glass house grown
Leaf length	**18.38**	14.2
Leaf width	**21.25**	14.31
Leaf thickness	16.34	16.92
Petiole length	**25.99**	14.25
Leaf area	33.09	**67.66**
Chlorophyll content	11.27	**16.47**
Dry weight	33.07	**38.13**
Specific leaf area	**19.52**	16.39

Table 3.3 Results of ANOVA for field and glass house grown leaves *Hippophae rhamnoides*

Variable	Field grown	Glass house grown
Leaf length	0.074	**0.018**
Leaf width	0.656	0.190
Leaf thickness	**0.000**	0.892
Petiole length	**0.000**	0.749
Leaf area	0.494	0.694
Chlorophyll content	**0.002**	**0.014**
Dry weight	**0.032**	0.168
Specific leaf area	**0.006**	0.668

Values shown significant at $p \leq 0.05$

Table 3.4 Result of regression analysis with altitude as explanatory variable and field grown leaves and glass house grown leaves of *Hippophae rhamnoides* as dependent variable

Variable	Field grown				Glass house grown			
	d.f.	b	R^2	*p*	d.f.	b	R^2	*P*
Leaf length	1,12	3.099	0.002	0.893	1,12	2.291	0.002	0.894
Leaf width	1,12	0.431	0.015	0.676	1,12	0.367	0.013	0.702
Leaf thickness	1,12	**-0.076**	**0.970**	**0.000**	1,12	0.204	0.179	0.132
Petiole length	1,12	0.025	0.215	0.095	1,12	0.189	0.176	0.135
Leaf area	1,12	1.254	0.006	0.790	1,12	1.712	0.177	0.134
Chlorophyll content	1,12	67.928	0.001	0.897	1,12	31.568	0.162	0.154
Dry weight	1,12	**-2.056**	**0.326**	**0.033**	1,12	2.112	0.027	0.577
Specific leaf area	1,12	**0.325**	**0.344**	**0.028**	1,12	0.623	0.213	0.096

Values shown siginificant at $p \leq 0.05$

Objective IV:

Gender-specific seasonal pattern and altitudinal variation in freeze tolerance responses in *Hippophae rhamnoides*

Woody plants in temperate regions are subjected to large seasonal variations in temperature, and have adapted to these conditions by evolving annual growth cycle that alternates between active shoot growth and vegetative dormancy and is closely synchronized with the seasonal changes (Li et al., 2003). Most plant species have evolved a degree of cold tolerance, the extent of which is typically dependent on combination of the minimum temperature experienced and the length of exposure to cold stress (Janská et al., 2010). The ability of plants to adapt to and survive freezing temperatures has many facets, which are often species specific, and are the result of the response to many environmental cues, rather than just low temperature (Gusta and Wisniewski, 2013). However, most of the freezing tolerance responses in plants are studied in controlled condition in growth chambers. There are significant differences between natural and artificial cold acclimation conditions and so plants that are cold acclimated in growth chamber may respond differently to those acclimated naturally (Robertson et al. 1994; Dhanaraj et al., 2007). Varying diurnal temperatures that produce mixed messages are common in the field in contrast to constant temperatures in a growth chamber. Any research designed to explore and understand cold hardiness should be verified in the context of the physiology, growth habit and life cycle of the plant grown under natural conditions (Gusta and Wisniewski, 2013). In dioecious plants, morphological and physiological adjustment to cold and freezing conditions may differ between male and female individuals due to greater reproductive effort in females. The cost of reproduction involves prioritization of resources in fruit development rather than in vegetative growth or protection in females. Not accounting for sexual variation could lead to incorrect assessment of a species response to freezing conditions. However, we are aware of

no studies which have addressed the relative importance of plant sex in freezing tolerance in natural conditions particularly in the trans-Himalaya.

Result

Gender-specific seasonal pattern in freezing tolerance

Seasonal pattern in acclimation and freezing tolerance is presented in Table 4.1. Electrolyte leakage and proline content in leaves and shoots varied significantly during the sampling period. Significant increase in electrolyte leakage was observed in leaves in mid October when the incidents of temperature below 5°C was observed on 25 days between mid September and mid October. The linear regression showed almost linear relation between electrolyte leakage and sampling period in both male (R^2=0.871) and female (R^2=0.882) leaves. However, electrolyte leakage in shoot remained constant throughout the sampling period and no linear relationship was observed in both the gender. Within each sampling period, there was no significant impact of gender on electrolyte leakage. The shoot electrolyte leakage was significantly higher than that of leaves in August and September. However, the opposite trend was observed in November and December in both the gender.

The proline content in leaves showed a significant increasing trend from August to October followed by a steady decline from November onwards (Table 4.1) in both the gender. However, the shoot proline content showed a steady increasing trend from August to December. Progression in season from August to December is related linearly to the increase in proline content in both male (R^2=0.967) and female (R^2=0.926) shoot. Leaves of male plants contained significantly higher proline (234±76 µM g^{-1}) than that of female (131±39 µM g^{-1}) in October ($P < 0.001$, Student's t-test). However, in shoot the proline content remained significantly higher in male than female from October to December (Table 4.1). Throughout the sampling period the proline content in shoot remained significantly higher than that of leaves except in October.

Altitudinal variation in freezing tolerance

Altitude of plant origin did not have significant impact on leaves and shoot electrolyte leakage of both male and female *H. rhamnoides* (Table 4.2, 4.3). Similarly, no increasing or decreasing trend was observed in leaf proline content with increasing altitude (Table 4.2). However, increasing altitude is related linearly to increase in proline content in both male (R^2=0.676) and female (R^2=0.858) shoot. Males collected from 3340 m altitude contained significantly higher proline in both leaves and shoot than that of females (P < 0.01, Student's t-test). The overall proline content in both leaves and shoot was significantly higher in male (112±77, 143±66 µM g^{-1}, respectively) than female (87±46, 119±82 µM g^{-1}, respectively). Within each sampling site, electrolyte leakage was significantly higher in leaves than shoot irrespective of the gender. Opposite trend was observed in proline content in both the gender.

Our results showed sexually different responses of *H. rhamnoides* to environmental cues in natural conditions. Females suffer more negative effects to freezing temperature than do males. Leaves developed freezing tolerance more quickly than shoot when exposed to chilling temperature. Therefore, when *H. rhamnoides* is planted in cold region, more effective protective measurements should be provided for females to improve their chance for survival.

Table 4.1 Seasonal pattern in electrolyte leakage and proline content as a measure of cold hardiness in *Hippophae rhamnoides*

Tissue	Date	Electrolyte Leakage (%)		Proline Content (µM g^{-1}FW)	
		Male	Female	Male	Female
Leaves	15 Aug	12.8±4.3^{a}	14.5±4.5^{a}	0.3±0.2 ^{a}	0.3±0.2^{a}
	15 Sept	12.4±10.9^{a}	11.6±4.2^{a}	26.8±17.1^{b}	32.7±23.5^{ab}
	15 Oct	38.5±11.8^{bc}	39.0±11.3^{b}	234.3±76.0^{d}***	130.7±38.9^{cd}
	15 Nov	65.1±13.6 ^{c}	62.8±6.6^{c}	71.3±23.5^{c}	69.1±20.9^{b}
	15 Dec	61.5±13.2^{c}	62.9±13.2^{c}	46.9±13.7^{bc}	31.8±7.1^{ab}
Shoot	15 Aug	35.4±17.6^{bc}	33.8±20.3^{bc}	0.6±0.4^{a}	0.5±0.4^{a}
	15 Sept	31.8±4.6^{b}	30.4±10.0^{b}	88.1±72.0^{c}	91.0±62.7^{bc}
	15 Oct	32.3±12.6^{bc}	36.5±14.8^{bc}	219.2±135.9^{d}***	108.5±77.7^{bc}
	15 Nov	35.2±8.6^{b}	33.2±16.9^{ab}	237.5±113.1^{d}*	164.0±120.4^{c}
	15 Dec	37.4±11.1^{bc}	37.1±9.5^{b}	361.7±158.6^{d}*	298.2±132.5^{d}

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by 2-sided Tukey's HSD

*value significantly higher than that of opposite sex at $p \leq 0.01$, *** value significantly higher than that of opposite sex at $p \leq 0.001$

Table 4.2 Altitudinal variation in electrolyte leakage and proline content in leaves as a measure of cold hardiness in *Hippophae rhamnoides* in December under natural field condition

Altitude (m asl)	Electrolyte Leakage (%)		Proline Content (µM g^{-1} FW)	
	Male	Female	Male	Female
3203±5.6	51.4±13.6^{a}	61.7±8.8^{b}	67.4±23.6^{a}	64.9±23.6^{a}
3239±5.0	59.6±8.6 ^{a}	63.0±9.4^{b}	145.8±103.7^{ab}	133.4±61.4^{b}
3260±4.6	65.9±10.5^{a}	58.0±11.2^{ab}	67.6±34.5^{a}	64.1±23.8^{a}
3340±8.7	64.7±7.5^{a}	62.9±14.5^{b}	177.5±108.0^{b}**	65.7±30.3^{a}
3464±23.9	52.3±12.1^{a}	42.8±11.7^{a}	119.3±23.7^{ab}	101.4±34.0^{ab}
3636±49.6	61.2±8.2^{a}	62.2±11.4^{b}	69.2±25.4^{a}	66.0±24.8 ^{a}
3812±24.8	54.1±14.5 ^{a}	58.7±10.3^{b}	134.6±86.1^{ab}	113.1±55.6^{ab}
Mean	58.4±11.9	58.5±12.6	111.6±77.4**	86.9±46.0

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by 2-sided Tukey's HSD

**Value significantly higher than that of opposite sex at $p \leq 0.01$.

Table 4.3 Altitudinal variation in electrolyte leakage and proline content in shoot as a measure of cold hardiness in *Hippophae rhamnoides* in December under natural field condition

Altitude (m asl)	Electrolyte Leakage (%)		Proline Content (µM g^{-1} FW)	
	Male	Female	Male	Female
3203±5.6	31.2±2.9[a]	31.3±3.7[a]	84.2±43.4[a]	59.6±56.6[a]
3239±5.0	38.8±6.2[a]	39.5±6.1[b]	113.6±48.2[ab]	73.0±43.6[a]
3260±4.6	33.0±16.6[a]	29.2±5.1[a]	121.3±47.4[ab]	96.6±66.1[abc]
3340±8.7	32.8±14.0[a]	33.2±9.0[ab]	175.1±75.8[b***]	75.0±19.3[ab]
3464±23.9	30.1±5.5[a]	26.6±3.7[a]	180.5±65.9[b]	163.8±53.8[bcd]
3636±49.6	31.1±4.7[a]	32.7±5.2[ab]	165.0±64.4[b]	166.3±98.8[cd]
3812±24.8	31.6±7.0[a]	32.1±4.7[ab]	159.6±59.0[ab]	195.7±90.8[d]
Mean	32.7±9.4	32.1±6.6	142.9±65.7***	118.6±81.5

Values represented as mean ± SD. For each column, different lowercase letters indicate significantly different at $p < 0.05$, as measured by 2-sided Tukey's HSD

***Value significantly higher than that of opposite sex at $p \leq 0.001$.

Objective V:

SSR markers based genetic diversity characterization and marker-trait correlation for antioxidant properties in male and female populations of *Hippophae rhamnoides*

A total of 157 flowering plant families and 959 flowering plant genera contribute to dioecy in the plant population. Population genetic diversity can affect the adaptation and tolerance of a species, especially diversity of sexual populations (Ilves et al., 2013).Most of the studies in male and female plant species have been confined to morphological and physiological differences. In general, sex-specific genetic diversity studies in dioecious plants including seabuckthorn is limited. While female bears berries that are rich in vitamin c and omega fatty acids, male populations are rich source of pharmaceutically important compounds. Moreover at present, the demand for planting material, berry, and leaf harvest is largely met by the wild populations that vary in quality and quantity of the active ingredient. Fulfillment of domestic and industrial demands of crops by harvesting wild resources may reduce the availability of the plant in nature. In view of the increasing threat to the natural populations, systematic crop improvement through the selection of important traits and plant breeding driven by genetic diversity is essential. These approaches can be helpful in better understanding the genetic diversity, quality control, germplasm conservation, and genetic improvement especially where other genetic information like the linkage is not available (Khub et a., 2014).In the present study, we made an attempt to delineate the genetic diversity study in 6 *H. rhamnoides* population (90 male and 90 female individuals) representing trans-Himalaya Ladakh region and subsequently these markers were correlated with antioxidant traits in seabuckthorn.

In house data on multiple genotypes available with our collaborating institute of Himalayan Bioresource Technology (Council of Scientific & Industrial Research) were mined for the identification of SSR.32 SSR markers(HR_T_1 to HR_T_32) was validated in 180 individuals belonging to six different populations. Antioxidant analysis of these individuals was previously performed (Objective:2) and data was used to correlate with SSR marker. Geographical location and collection site are provided in (Table 5.1) and (Fig. 5.1).

Result:

Thirty two SSR markers used in our study, all of them were found to be polymorphic and generated 77 alleles. Overall analysis revealed that the genetic diversity estimators were high. However, differences were not significant between male and female: percentage polymorphic loci 92.19% ♂ and 79.69 ♀, Nei's gene diversity 1.565 ♂ and 1.513 ♀, and Shannon's information index 0.493 ♂ and 0.432 ♀ (Table 5.2). Neighbour joining clustering analysis clustered 180 individuals from 6 populations (Table 5.1, Fig 5.1) of the trans-Himalayan region of Ladakh into four groups (Fig 5.2) wherein the geographically separated populations were found to be genetically diverse as compared to the populations of same belts that showed an admixture behavior. Male and female individuals did not grouped separately. The pairwise differentiation was moderate to low (ϕ=0.010 to 0.217) based on geographical location (Table 5.4) and showed significant amount of gene flow (Nm=3.37)which was further confirmed by AMOVA where within population genetic variance was 88% and between population genetic variance was 12% (Table 5.3). A Multiple Regression Analysis used to correlate markers with antioxidant activity illustrated that eight SSR markers (HR_T_1, HR_T_2, HR_T_3, HR_T_4, HR_T_5, HR_T_6, HR_T_8, and HR_T_9) with 29 alleles exhibited significant ($p < 0.001, 0.01, 0.05$) correlation (positive or negative) with TPC, FRAP, and DPPH assay (Fig 5.5). Three markers were correlated only with male and six markers correlated only with female individuals (Fig 5.5).

These results suggest that natural distribution played an important role in shaping the tree in genetic diversity of seabuckthorn and it appears that sex differentiation may occurred earlier than genetic differentiation thus male and female species did not grouped in different clusters which was in consistent with Zhai et al. (2016). Further, it is recommended that markers correlated with antioxidants along with diverse populations can be utilized for selection and breeding programs in future where no other tools like QTL are available.

Table 5.1. Details of geographical location of *H. rhamnoides* from the Ladakh region.

Sampling Locations	Population ID	Latitude (N)	Longitude (E)	Altitude (m) ASL	Sample size
Spituk	SPT	34º07′7″	77º30′4″	3203±5.6	30 (1-15 ♂, 15-30 ♀)
Chuchot	CHU	34º05′4″	77º35′9″	3239±5.0	30 (31-45 ♂, 46-60 ♀)
Shey	SHY	34º04′1″	77º37′7″	3260±4.6	30 (61-75 ♂, 76-90 ♀)
Phyang	PHY	34º11′5″	77º30′1″	3636±19.6	30 (91-105 ♂, 106-120 ♀)
Horzey	HOR	34º12′1″	77º35′3″	3812±14.8	30 (121-135 ♂, 136-150 ♀)
Sakti	SKT	34º02′1″	77º48′6″	3885±17.3	30 (151-165 ♂, 166-180 ♀)

Fig 5.1: Map showing the location of the populations studied (source: Google Maps) (arrow represents sampling site)

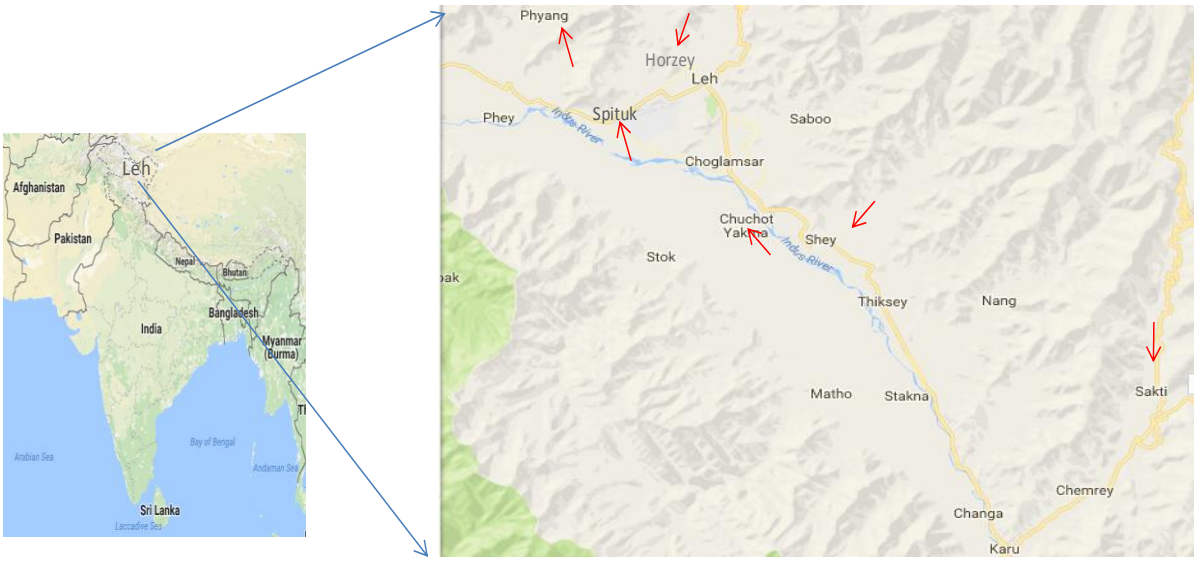

Table 5.2: Estimates of genetic diversity in male, female and 6 plantations of *H.rhamnoides.*

Sex	Population	% P	Na	Ne	I	h	uh
	Spituk	84.38	1.750	1.526	0.462	0.310	0.332
	Chuchot	90.63	1.844	1.548	0.478	0.320	0.343
	Shey	87.50	1.813	1.564	0.482	0.326	0.349
Male	Phyang	96.88	1.938	1.628	0.541	0.365	0.391
	Horzey	96.88	1.938	1.595	0.517	0.346	0.371
	Sakti	96.88	1.938	1.525	0.478	0.314	0.337
	Mean	**92.19**	**1.870**	**1.565**	**0.493**	**0.330**	**0.354**
	Spituk	78.13	1.656	1.472	0.410	0.274	0.293
	Chuchot	68.75	1.500	1.444	0.375	0.253	0.271
	Shey	78.13	1.719	1.506	0.437	0.295	0.316
Female	Phyang	81.25	1.719	1.602	0.482	0.333	0.357
	Horzey	84.38	1.750	1.505	0.429	0.287	0.308
	Sakti	87.50	1.844	1.547	0.468	0.315	0.338
	Mean	**79.69**	**1.698**	**1.513**	**0.434**	**0.293**	**0.314**

%P, percentage of polymorphic loci; **n**a, no. of different alleles; **n**e, no. of effective alleles; **h**, gene diversity; **I**, Shannon's information index; *uh*, unbiased diversity.

Fig 5.2: **Bootstrapped neighbour–joining (NJ) tree of 180 *H.rhamnoides* accessions using 32 SSR markers.**

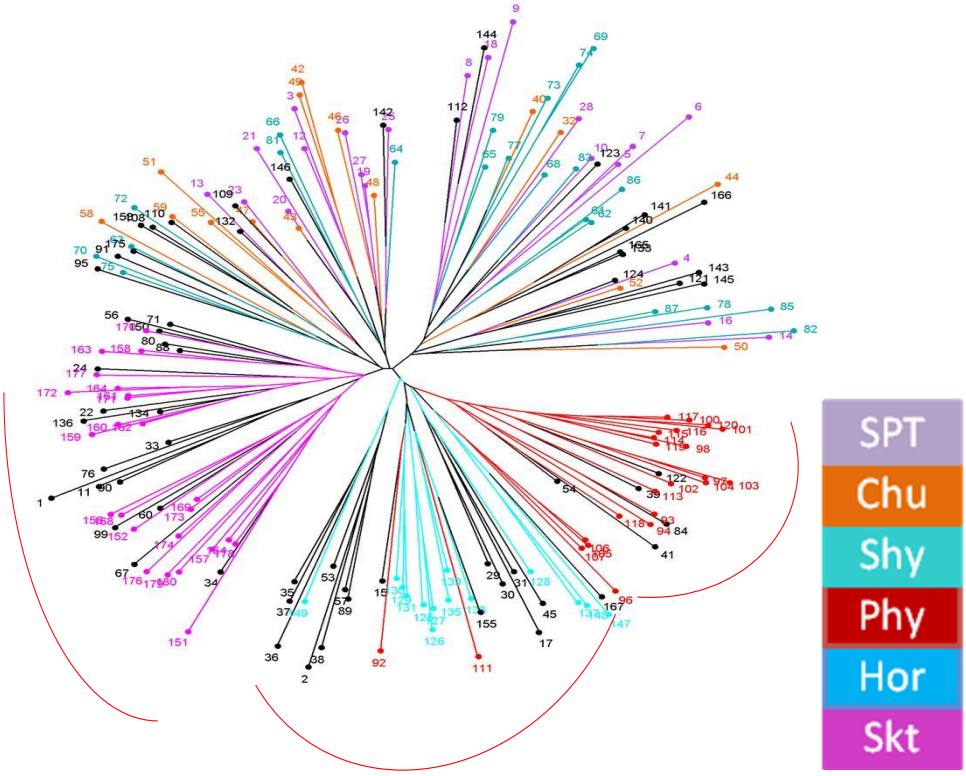

Table 5.3. Total genetic variance analysis (AMOVA) among and within populations of *H. rhamnoides* based on binary data obtained from SSR results.

Source of variation	DF	SS	Variance components	% of total Variance	ϕPT	P value
Among population	11	177.54	1.196	12	0.199	0.000
Within population	168	897.20	11.561	88	-	-

DF,degrees of freedom; SS, sum of squares; *P*, level of significance for estimation of genetic variation based on 9999 permutations.

Table 5.4. Population pairwise ϕPT in *H. rhamnoides* ten plantations

	SPT_M	SPT_F	Chu_M	Chu_F	Shey_M	Shey_F	Phy_M	Phy_F	Hor_M	Hor_F	Skt_M	Skt_F
SPT_M	0.000											
SPT_F	0.077	0.000										
Chu_M	**0.037**	0.013	0.000									
Chu_F	0.085	**0.015**	0.034	0.000								
Shey_M	0.086	0.089	0.061	0.046	0.000							
Shey_F	0.063	0.068	0.052	0.040	0.000	0.000						
Phy_M	0.105	0.170	0.128	0.175	0.192	0.192	0.000					
Phy_F	0.172	0.126	0.116	0.126	0.148	0.125	0.057	0.000				
Hor_M	0.138	0.121	0.112	0.135	0.142	0.120	0.123	0.126	0.000			
Hor_F	0.103	0.095	0.103	0.139	0.115	0.130	0.152	0.114	0.029	0.000		
Skt_M	0.186	0.189	0.133	0.159	0.162	0.152	**0.203**	0.191	0.089	0.108	0.000	
Skt_F	0.149	0.150	0.105	0.138	0.143	0.144	0.191	**0.217**	0.106	0.117	0.018	0.000

Table 5.5: SSR markers/alleles correlation with antioxidant activity in *H.rhamnoides* as revealed by stepwise MRA

Antioxidant Activity	SSR markers	Male			Female		
		Standardized beta coefficients	t value	p value	Standardized beta coefficients	t value	p value
Total phenolics content	HR_T_2D	-	-	-	-0.278	-2.711	0.008
	HR_T_4B	-0.220	-2.188	0.037	-	-	-
	HR_T_5A	-	-	-	-0.255	-2.479	0.015
	HR_T_5B	-	-	-	-0.337	-3.357	0.001
	HR_T_6B	-0.118	-1.779	0.015	-	-	-
	HR_T_8B	-	-	-	0.212	2.033	0.045
	HR_T_8D	-	-	-	-0.285	-2.789	0.006
	HR_T_9D	-	-	-	0.223	2.148	0.034
	HR_T_9E	0.211	2.112	0.046	0.235	2.267	0.026
FRAP reducing antioxidant assay	HR_T_1A	-2.453	-2.355	0.021	-	-	-
	HR_T_1B	-	-	-	-0.252	-2.244	0.017
	HR_T_2B	0.308	3.042	0.003	0.280	2.736	0.008
	HR_T_2D	-0.210	-2.010	0.047	-0.325	-3.266	0.002
	HR_T_3C	-	-	-	-0.218	-2.100	0.039
	HR_T_3D	-	-	-	0.255	2.162	0.033
	HR_T_4B	-0.250	-2.427	0.017	-	-	-
	HR_T_5D	-0.340	-3.369	0.001	-0.415	-4.275	0.000
	HR_T_6A	-0.212	-2.035	0.045	-	-	-
	HR_T_9C	-0.327	-3.243	0.002	-	-	-
	HR_T_9D	-	-	-	0.259	2.513	0.014
	HR_T_9E	0.290	2.845	0.006	-	-	-
DPPH Assay	HR_T_1B	-	-	-	0.229	2.210	0.030
	HR_T_2C	-	-	-	-0.251	-2.433	0.017
	HR_T_2D	-	-	-	0.385	3.912	0.000
	HR_T_3C	-	-	-	0.218	2.091	0.039
	HR_T_4B	0.257	2.495	0.014	0.255	2.168	0.033
	HR_T_5D	-	-	-	0.336	3.343	0.001
	HR_T_6A	0.320	3.168	0.002	-	-	-
	HR_T_9D	-	-	-	-0.026	-1.977	0.051

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